JVI Journals.ASM.org

Complete Genome Characterization of Recent and Ancient Belgian Pig Group A Rotaviruses and Assessment of Their Evolutionary Relationship with Human Rotaviruses

Sebastiaan Theuns,[a] Elisabeth Heylen,[b] Mark Zeller,[b] Inge D. M. Roukaerts,[a] Lowiese M. B. Desmarets,[a] Marc Van Ranst,[b] Hans J. Nauwynck,[a] Jelle Matthijnssens[b]

Ghent University, Faculty of Veterinary Medicine, Department of Virology, Parasitology and Immunology, Laboratory of Virology, Merelbeke, Belgium[a]; KU Leuven-University of Leuven, Department of Microbiology and Immunology, Laboratory for Clinical and Epidemiological Virology, Rega Institute for Medical Research, Leuven, Belgium[b]

ABSTRACT

Group A rotaviruses (RVAs) are an important cause of diarrhea in young pigs and children. An evolutionary relationship has been suggested to exist between pig and human RVAs. This hypothesis was further investigated by phylogenetic analysis of the complete genomes of six recent (G2P[27], G3P[6], G4P[7], G5P[7], G9P[13], and G9P[23]) and one historic (G1P[7]) Belgian pig RVA strains and of all completely characterized pig RVAs from around the globe. In contrast to the large diversity of genotypes found for the outer capsid proteins VP4 and VP7, a relatively conserved genotype constellation (I5-R1-C1-M1-A8-N1-T7-E1-H1) was found for the other 9 genes in most pig RVA strains. VP1, VP2, VP3, NSP2, NSP4, and NSP5 genes of porcine RVAs belonged to genotype 1, which is shared with human Wa-like RVAs. However, for most of these gene segments, pig strains clustered distantly from human Wa-like RVAs, indicating that viruses from both species have entered different evolutionary paths. However, VP1, VP2, and NSP3 genes of some archival human strains were moderately related to pig strains. Phylogenetic analysis of the VP6, NSP1, and NSP3 genes, as well as amino acid analysis of the antigenic regions of VP7, further confirmed this evolutionary segregation. The present results also indicate that the species barrier is less strict for pig P[6] strains but that chances for successful spread of these strains in the human population are hampered by the better adaptation of pig RVAs to pig enterocytes. However, future surveillance of pig and human RVA strains is warranted.

IMPORTANCE

Rotaviruses are an important cause of diarrhea in many species, including pigs and humans. Our understanding of the evolutionary relationship between rotaviruses from both species is limited by the lack of genomic data on pig strains. In this study, recent and ancient Belgian pig rotavirus isolates were sequenced, and their evolutionary relationship with human Wa-like strains was investigated. Our data show that Wa-like human and pig strains have entered different evolutionary paths. Our data indicate that pig P[6] strains form the most considerable risk for interspecies transmission to humans. However, efficient spread of pig strains in the human population is most likely hampered by the adaptation of some crucial viral proteins to the cellular machinery of pig enterocytes. These data allow a better understanding of the risk for direct interspecies transmission events and the emergence of pig rotaviruses or pig-human reassortants in the human population.

Enteric diseases in pigs are mostly encountered during two critical time points: the suckling period and after weaning. Several pathogens are frequently involved in the pathogenesis of piglet diarrhea, including rotavirus, porcine epidemic diarrhea virus, transmissible gastroenteritis virus, *Escherichia coli*, *Clostridium perfringens*, *Salmonella* spp., *Brachyspira* spp., and *Isospora suis*. Furthermore, coinfections between different rotaviruses and other pathogens can be found frequently in diarrheic pigs (1).

Rotaviruses are a major cause of diarrhea in many species, including pigs and humans. Five official rotavirus species (A to E) and 3 tentative species (F to H) have been established based on nucleotide similarities of the VP6-encoding genes (2). Species A, B, and C are frequently isolated from feces of diarrheic and nondiarrheic pigs, whereas rotavirus group E was detected in a pig fecal sample only once (3). Rotavirus species H also seems to be epizootiologically important in pigs, but its role in the pathogenesis of piglet diarrhea remains to be elucidated (4, 5). Nevertheless, group A rotaviruses (RVAs) are considered the most important rotavirus species in pigs and humans.

The RVA genome possesses 11 segments of double-stranded RNA (dsRNA), encoding 6 structural (VP1 to VP4, VP6, and VP7) and 6 nonstructural (NSP1 to NSP6) proteins. Genes encoding outer capsid proteins VP7 and VP4 are of utmost importance, because these proteins can induce neutralizing antibodies (6–8). For VP7 and VP4, 27 G and 37 P genotypes, respectively, have

Received 1 September 2014 **Accepted** 23 October 2014

Accepted manuscript posted online 5 November 2014

Citation Theuns S, Heylen E, Zeller M, Roukaerts IDM, Desmarets LMB, Van Ranst M, Nauwynck HJ, Matthijnssens J. 2015. Complete genome characterization of recent and ancient Belgian pig group A rotaviruses and assessment of their evolutionary relationship with human rotaviruses. J Virol 89:1043–1057. doi:10.1128/JVI.02513-14.

Editor: D. S. Lyles

Address correspondence to Sebastiaan Theuns, Sebastiaan.Theuns@UGent.be.

H.J.N. and J.M. are co-senior authors.

doi:10.1128/JVI.02513-14

TABLE 1 GenBank accession numbers of pig strains analyzed in the present study

Gene	GenBank accession no. for pig strain:						
	12R002 (G5P[7])	12R005 (G4P[7])	12R006 (G3P[6])	12R022 (G2P[27])	12R041 (G9P[13])	12R046 (G9P[23])	RV277 (G1P[7])
VP1	KM820695	KM820696	KM820697	KM820698	KM820699	KM820700	KM820701
VP2	KM820702	KM820703	KM820704	KM820705	KM820706	KM820707	KM820708
VP3	KM820709	KM820710	KM820711	KM820712	KM820713	KM820714	KM820715
VP4	KM820716	KM820717	KM820719	KM820721	KM820718	KM820720	KM820722
VP6	KM820723	KM820724	KM820725	KM820726	KM820727	KM820728	KM820729
VP7	KM820730	KM820731	KM820732	KM820733	KM820734	KM820735	KM820736
NSP1	KM820737	KM820738	KM820739	KM820740	KM820741	KM820742	KM820743
NSP2	KM820667	KM820668	KM820669	KM820670	KM820671	KM820672	KM820673
NSP3	KM820674	KM820675	KM820676	KM820677	KM820678	KM820679	KM820680
NSP4	KM820681	KM820682	KM820683	KM820684	KM820685	KM820686	KM820687
NSP5	KM820688	KM820689	KM820690	KM820691	KM820692	KM820693	KM820694

been recognized in many host species so far (8, 9). In feces from pigs, 12 different G genotypes (G1 to G6, G8 to G12, and G26) and 13 different P genotypes (P[1], P[5] to P[8], P[11], P[13], P[19], P[23], P[26], P[27], P[32], and P[34]) have been found worldwide (8, 10, 11). Recently, the circulation of genotypes G2, G3, G4, G5, G9, and G11 in combination with P[6], P[7], P[13], P[23], or P[27] was demonstrated in Belgium (1). Characterization of the oldest Belgian pig RVA strain (RVA/Pig-tc/BEL/RV277/1977/G1P[7]), isolated in 1977, identified the rare G1P[7] genotype combination. This strain was isolated from a pool of watery feces of three 2-day-old piglets that were kept in isolation for experimental purposes. Four days after isolation, they showed symptoms of watery diarrhea and dehydration and finally died (1, 12). The vast genetic diversity seen among genes encoding the outer capsid proteins of pig strains is in contrast with the relatively restricted number of G/P combinations found in human RVA strains.

Due to the segmented nature of the rotavirus genome, a full genome-based classification system has been established for RVA. As such, each gene is abbreviated by a letter followed by a number designating the genotype. The genotype constellation of each RVA strain can be presented in the form Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx, which indicates the genotypes of VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5 (7). Complete genome analysis of ancient cell-culture-grown pig RVA strains (RVA/Pig-tc/USA/OSU/1977/G5P[7], RVA/Pig-tc/USA/Gottfried/1983/G4P[6], RVA/Pig-tc/MEX/YM/1983/G11P[7], and RVA/Pig-tc/IND/RU172/2002/G12P[7]), possessing different G/P combinations for VP7 and VP4, in most cases identified an I5-R1-C1-M1-A8-N1-T1-E1-H1 genetic backbone for the other viral genes (13, 14).

Most human RVA strains carry one of the two major genotype constellations, Gx-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1 and G2-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2, and are called Wa-like and DS-1-like strains, respectively (15). Pig and human Wa-like RVA strains are suggested to possess a common evolutionary ancestor, because genotype 1 genes can be found in strains from both species (6). However, the restricted number of complete genomes of contemporary wild-type pig RVA strains makes it difficult to come to sound conclusions. To obtain better insights into the evolutionary relationship between pig and human RVAs, more completely characterized pig RVA genomes are necessary. A number of pig RVA strains from Canada, Thailand, South Korea, and Italy, possessing different VP7/VP4 genotype combinations, have been characterized to date. Most of these strains also possess an I5-C1-R1-M1-A8-N1-T1-E1-H1 genotype constellation for the 9 remaining genes (16–19). However, the T7 genotype for NSP3 has frequently been detected in these studies as well (17, 18). A rarity was the detection of the dual I5+I14 genotype for VP6 in a G2P[34] strain from Canada, which also possessed the rarely detected E9 genotype for NSP4 (17). VP7 and VP4 genes of Belgian strains were recently characterized, but information about the genetic composition of the other 9 genes of these Belgian strains was lacking. One exception is the completely characterized genome of strain RVA/Human-wt/BEL/BE2001/2009/G9P[6], isolated from a Belgian child, which has been suggested to represent a pig-to-human interspecies transmission event (20).

In the present study, we aimed to characterize the complete genomes of a selection of Belgian pig RVAs possessing widely diverse VP7 and VP4 genotypes. In addition, the historic strain RV277 (G1P[7]) was included. An increased availability of complete pig RVA genomes not only will provide useful information for the development of preventive measures against pig RVA infections but also will lead to a better understanding of the evolutionary relatedness between pig and human RVAs. This will facilitate recognition of newly emerging or reassorted pig-derived strains in humans.

MATERIALS AND METHODS

Selection of rotavirus strains. Fecal samples from diarrheic and asymptomatic piglets were collected on Belgian pig farms in 2012, as reported before (1). Six contemporary pig RVA strains were included for complete genome analysis in the present study (Table 1). In the present study, we aimed to include strains with representative G/P combinations from the previous study and to cover at least all unique VP4 and VP7 genotypes.

Five samples were obtained from diarrheic pigs (samples 12R002, 12R005, 12R006, 12R041, and 12R0046), whereas one sample (12R022) was obtained from a nondiarrheic pig. From these, fecal suspensions were used as starting material for RNA isolation and reverse transcriptase PCR (RT-PCR). In addition, a historic Belgian pig RVA strain (isolated in 1977) was also completely characterized to analyze if this strain was genetically similar to contemporary pig strains. Cell culture supernatant of the first passage of strain RV277 in MA104 cells was used as starting material for RNA extraction using a QIAamp viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and RNA was stored at −70°C.

RT-PCR. RNA was denatured at 95°C for 2 min and immediately chilled on ice. For the shorter gene segments (VP6, VP7, NSP2, NSP3, NSP4, and NSP5), reaction mixtures consisted of 5 μl of RNA, 5 μl of 5×

Qiagen OneStep RT-PCR buffer, 1 μl of deoxynucleoside triphosphate (dNTP) mix, 1.5 μl (each) of forward and reverse primers (830 nM), 1 μl of Qiagen OneStep RT-PCR enzyme mix, and nuclease-free water in a total volume of 25 μl. Reaction volumes were scaled up to 50 μl for the longer gene segments (VP1, VP2, VP3, VP4, and NSP1). Primers used for RT-PCR amplification of the complete gene segments will be made available upon request.

Reverse transcription was performed at 50°C for 30 min, followed by *Taq* polymerase activation at 94°C for 15 min and then 35 cycles of amplification. For the longer fragments, denaturation was performed at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 6 min. For the shorter fragments, denaturation was performed at 94°C for 30 s, annealing at 47°C for 30 s, and extension at 72°C for 2 min. A final extension step was performed for 10 min at 72°C. Afterwards, 9 μl of PCR product was mixed with 1 μl of loading buffer and loaded onto polyacrylamide gels. Electrophoresis was performed at 200 V for 36 min, followed by ethidium bromide staining of the gels for detection of positive samples.

Sanger sequencing. Five microliters of PCR-positive sample was treated with 1 μl of ExoSAP-IT For PCR Product Cleanup reagent (Affymetrix, Santa Clara, CA) and sequenced with an ABI Prism BigDye Terminator cycle sequencing reaction kit (ABI Prism 3130xl; Applied Biosystems), using forward and reverse primers. Next, remaining parts of the coding regions were further covered by primer-walking sequencing. The 5′- and 3′-terminal sequences were obtained using a modified version of the single-primer amplification method (21). Briefly, a modified amino-linked oligonucleotide (TGP-Linker; 5′-PO_4-TTCCTTATGCAGCTGAT CACTCTGTGTCA-spacer-NH_2-3′) was linked to the 3′ end of both strands of denatured RNA by using T4 RNA ligase (Promega, Leiden, The Netherlands). Next, RT-PCR was performed using an internal gene-specific primer and primer TGP-3Out. First, reverse transcription was performed at 45°C for 30 min, followed by PCR activation at 95°C for 15 min. During a period of 45 min, the temperature was gradually lowered from 83°C to 60°C, the temperature was then held for 10 min at 72°C, and 40 cycles of amplification followed, with cycles of 94°C for 45 s, 45°C for 45 s, and 70°C for 1 min. A final extension was performed at 70°C for 7 min. Products were separated by polyacrylamide gel electrophoresis for 18 min at 200 V, followed by sequencing.

Phylogenetic analysis. Sequence analysis was performed using 4Peaks (Mekentosj, Amsterdam, The Netherlands), and genotypes for all genes were determined using BLAST and RotaC$^{2.0}$ (22). Multiple-sequence alignments were performed using the ClustalW plug-in in MEGA 5.2.2, followed by manual editing.

For each gene, maximum likelihood phylogenetic trees were constructed, and bootstrap analysis was set at 500 replicates. Substitution models were determined for each gene separately in MEGA 5.2.2. Pairwise distances were calculated using the *P*-distance model in MEGA 5.2.2.

Nucleotide sequence accession numbers. Nucleotide sequences were uploaded to GenBank, and accession numbers are provided in Table 1.

RESULTS

Genotype constellation of Belgian pig group A rotaviruses. The genotype constellations of all Belgian pig RVAs are shown in Table 2. Whereas a wide variety of G/P genotypes was detected for the VP7- and VP4-encoding genes, the following constellation was dominantly found for the other genes of Belgian pig rotaviruses: Gx-P[x]-I5-R1-C1-M1-A8-N1-T7-E1-H1. For the inner capsid protein of ancient strain RV277, an I1 genotype was encountered. Although the T7 genotype for NSP3 was found in the majority ($n = 4/7$) of Belgian strains, the T1 genotype was also encountered in 3 of 7 RVA strains. Furthermore, an E9 genotype was found for NSP4 in nondiarrheic strain 12R022.

Phylogenetic analysis of genes encoding structural proteins. (i) Outer capsid proteins (VP7 and VP4). A maximum likelihood phylogenetic tree was constructed for VP7, using the general time-reversible model with gamma distribution and invariant sites. Belgian pig strains of the present study were assigned to 6 different G genotypes (G1, G2, G3, G4, G5, and G9) for the glycosylated outer capsid protein VP7 (Fig. 1). Overall, a high genetic diversity was demonstrated by phylogenetic analysis of the VP7-encoding genes. It is interesting that for genotypes which are shared between pig and human RVA strains, such as G1, G2, G3, G4, and G9, the genetic distance between pig and human strains was high. As an example, ancient strain RV277 clustered distantly from contemporary human G1 strains (84.7 to 86.8% nucleotide similarity). However, this strain was most closely related to the rare human G1P[6] strain RVA/Human-tc/JPN/AU-19/1997/G1P[6] (94.8%). This strain belonged to an animal-like cluster within the G1 genotype, but still RV277 clustered distantly from other pig and bovine G1 strains (85.1 to 90.4%). Moreover, pig G2 strains clustered even more distantly from human strains (78.7 to 81.0%). One strain, RVA/Pig-wt/CAN/F8-4/2006/G2P[6]/[7], clustered between pig (79.2 to 80.1%) and human (80.4 to 81.5%) RVA strains. Strain 12R022 was most related to a Spanish G2 strain, RVA/Pig-wt/ESP/34461-4/2003/G2P[23] (93.0%). While G3 strains can also be encountered in different species, pig G3 strains were slightly more related to human G3 strains than to G3 strains from cats and horses (79.2 to 81.9%), albeit at a high genetic distance (86.1 to 88.8%). Different lineages within G4 have also been described, and contemporary human G4 strains have been shown to be genetically distinct from pig G4 strains. As an example, Belgian strain 12R005 clustered distinctly from contemporary human G4 strains (83.0 to 83.7%). However, this strain clustered with pig-like human G4P[6] RVA strains from Hungary (85.0 to 87.2%). The highest relatedness between pig and human strains was demonstrated within the G9 genotype. The two included pig G9 strains were 93.2% similar to contemporary human G9 strains from the major subcluster of lineage III. Both G9 strains were also highly related to the Belgian pig-like human strain RVA/Human-wt/BEL/BE2001/2009/G9P[6] (95.9 to 96.2%). Furthermore, genetic diversity within the G5 genotype was high, and strain 12R002 clustered most closely to Korean strain RVA/Pig-wt/KOR/187-1/2006/G5P[7] (87.1%).

For construction of the VP4 maximum likelihood tree, the Hasegawa-Kishino-Yano model with gamma distribution and invariant sites was used. Phylogenetic analysis of the encoding genes showed that some genotypes clustered more closely than others (Fig. 1). As an example, one cluster was composed of the major human genotypes P[4] and P[8] and genotypes P[6] and P[19], which have been detected in both pigs and humans. Within P[6], Belgian strain 12R006 clustered distantly from the contemporary human P[6] strains (81.1 to 82.3%). In contrast, the Belgian pig strain was most closely related to pig-like human strains from Belgium and Hungary (90.6 to 97.0%). The other P genotypes, which are mainly encountered in animals, made up several other groups of genotypes. Three Belgian P[7] strains were included in the present study. Contemporary strains 12R002 and 12R005 clustered together (90.6%) but were distantly related to ancient strain RV277 (85.6 to 88.6%), which clustered most closely to ancient strain OSU and a Korean strain (95.0%). The genetic diversity in the P[13] genotype was apparent by the presence of multiple lineages. Intragenotypic heterogeneity was also high in genotype P[23]. Belgian strain 12R046 clustered most closely to strain RVA/Pig-tc/CHN/NMTL/2008/G9P[23], from China (93.2%). One P[27] strain, 12R022, was included in the present study, but only a

TABLE 2 Genotype constellations of completely characterized pig RVA strains[a]

Strain	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5	Reference
RVA/Pig-tc/BEL/RV277/1977/G1P[7]	G1	P[7]	I1	R1	C1	M1	A8	N1	T7	E1	H1	This study
RVA/Pig-wt/BEL/12R022/2012/G2P[27]	G2	P[27]	I5	R1	C1	M1	A8	N1	T7	E9	H1	This study
RVA/Pig-wt/BEL/12R006/2012/G3P[6]	G3	P[6]	I5	R1	C1	M1	A8	N1	T1	E1	H1	This study
RVA/Pig-wt/BEL/12R005/2012/G4P[7]	G4	P[7]	I5	R1	C1	M1	A8	N1	T7	E1	H1	This study
RVA/Pig-wt/BEL/12R002/2012/G5P[7]	G5	P[7]	I5	R1	C1	M1	A8	N1	T1	E1	H1	This study
RVA/Pig-wt/BEL/12R041/2012/G9P[13]	G9	P[13]	I5	R1	C1	M1	A8	N1	T7	E1	H1	This study
RVA/Pig-wt/BEL/12R046/2012/G9P[23]	G9	P[23]	I5	R1	C1	M1	A8	N1	T1	E1	H1	This study
RVA/Pig-wt/CAN/F8-4/2006/G2P[6]/[7]	G2	P[6/7]	I5	R1	C1	M1	A8	N1	T7	E1	H1	17
RVA/Pig-wt/CAN/AB82/2006/G2P[34]	G2	P[34]	I5/I14	R1	C1	M1	A8	N1	T7	E9	H1	17
RVA/Pig-tc/VEN/A131/1988/G3P[7]	G3	P[7]	I5	R1	C2	M1	A1	N1	T1	E1	H1	6
RVA/Pig-wt/THA/CMP29/08/2008/G3P[13]	G3	P[13]	I5	R1	C1	M1	A8	N1	T1	E1	H1	19
RVA/Pig-wt/THA/CMP40/08/2008/G3P[23]	G3	P[23]	I5	R1	C1	M1	A8	N1	T1	E1	H1	19
RVA/Pig-wt/THA/CMP48/08/2008/G3P[23]	G3	P[23]	I5	R1	C1	M1	A8	N1	T1	E1	H1	19
RVA/Pig-tc/USA/Gottfried/1983/G4P[6]	G4	P[6]	I1	R1	C1	M1	A8	N1	T1	E1	H1	13
RVA/Pig-tc/USA/OSU/1977/G5P[7]	G5	P[7]	I5	R1	C1	M1	A1	N1	T1	E1	H1	13
RVA/Pig-tc/KOR/PRG9121/2006/G9P[7]	G9	P[7]	I5	R1	C1	M1	A8	N1	T1	E1	H1	16
RVA/Pig-wt/CAN/F7-4/2006/G9P[7]/[13]	G9	P[7/13]	I5	R1	C1	M1	A8	N1	Tx	E1	H1	17
RVA/Pig-wt/THA/CMP45/08/2008/G9P[23]	G9	P[23]	I5	R1	C1	M1	A8	N1	T7	E1	H1	19
RVA/Pig-tc/KOR/PRG921/2006/G9P[23]	G9	P[23]	I5	R1	C1	M1	A8	N1	T1	E1	H1	16
RVA/Pig-tc/KOR/PRG9235/2006/G9P[23]	G9	P[23]	I5	R1	C1	M1	A8	N1	T1	E1	H1	16
RVA/Pig-tc/KOR/PRG942/2006/G9P[23]	G9	P[23]	I5	R1	C1	M1	A8	N1	T1	E1	H1	16
RVA/Pig-wt/ITA/2CR/2009/G9P[23]	G9	P[23]	I5	R1	C1	M1	A8	N1	T7	E1	H1	18
RVA/Pig-wt/ITA/3BS/2009/G9P[23]	G9	P[23]	I5	R1	C1	M1	A8	N1	T1	E1	H1	18
RVA/Pig-wt/ITA/7RE/2009/G9P[23]	G9	P[23]	I5	R1	C1	M1	A8	N1	T1	E1	H1	18
RVA/Pig-tc/CHN/NMTL/2008/G9P[23]	G9	P[23]	I5	R1	C1	M1	A8	N1	T1	E1	H1	49
RVA/Pig-tc/MEX/YM/1983/G11P[7]	G11	P[7]	I5	R1	C1	M1	A8	N1	T1	E1	H1	13
RVA/Pig-tc/VEN/A253/1988/G11P[7]	G11	P[7]	I5	R1	C2	M1	A1	N1	T1	E1	H1	6
RVA/Pig-wt/CAN/F6-4/2006/G11P[13]	G11	P[13]	Ix	Rx	Cx	M1	A8	N1	T7	Ex	Hx	17
RVA/Pig-tc/IND/RU172/2002/G12P[7]	G12	P[7]	I5	R1	C1	M1	A1	N1	T1	E1	H1	14

[a] Belgian strains are shown in bold. Color code: blue, typical pig genotype; green, Wa-like genogroup; red, DS-1-like genogroup.

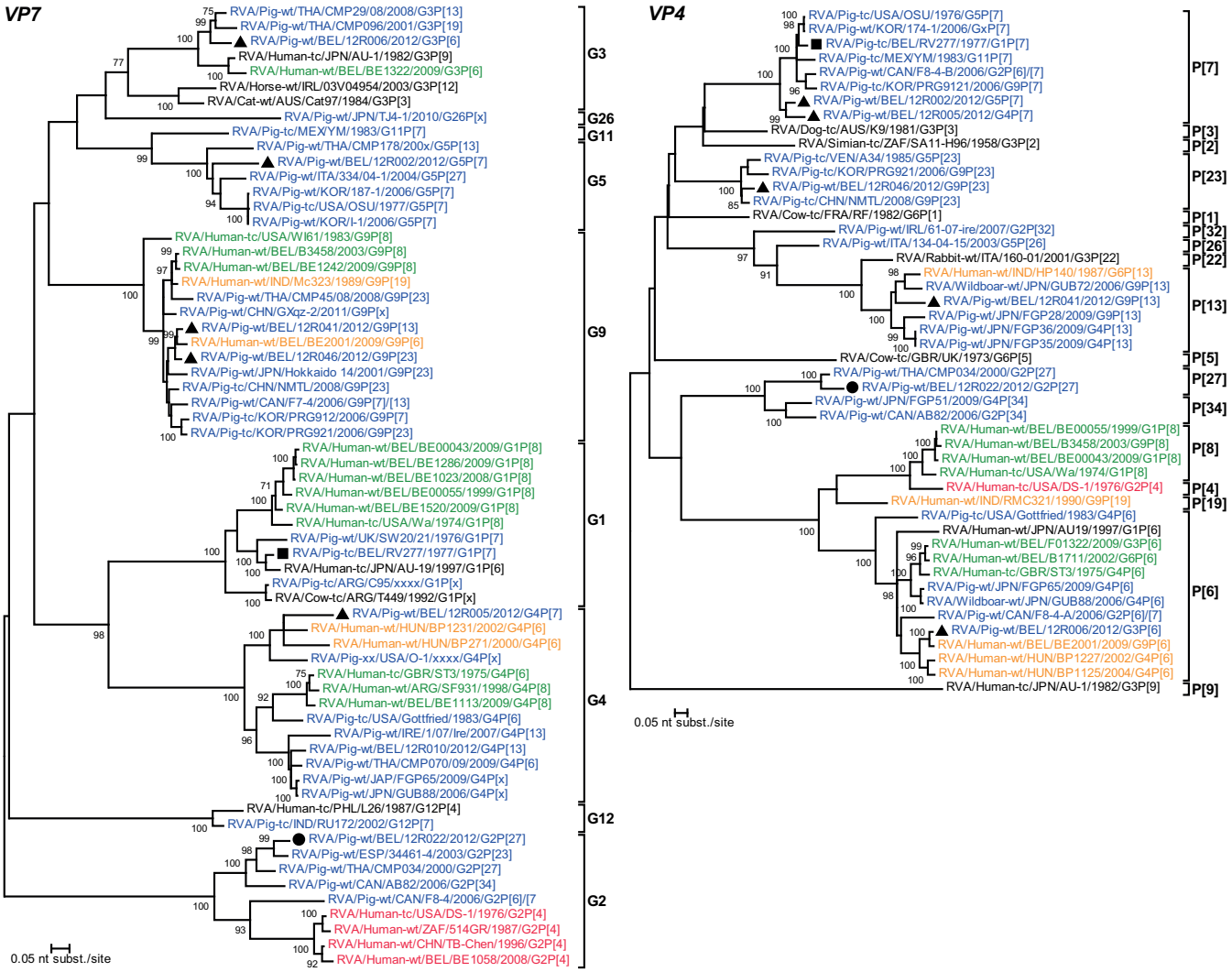

FIG 1 Maximum likelihood phylogenetic trees based on coding sequences of VP7 and VP4 genes. Bootstrap values ($n = 500$ replicates) of <70% are not shown. Pig strains are shown in blue, whereas human Wa-like strains are shown in green. Human DS-1-like strains are shown in red. Strains shown in orange are results of suspected interspecies transmission events between pigs and humans. Belgian pig strains are marked with triangles (contemporary and diarrheic), circles (contemporary and nondiarrheic), or squares (diarrheic and historic).

partial VP8* sequence could be obtained. This strain was relatively closely (89.9%) related to strain RVA/Pig-wt/THA/CMP034/2000/G2P[27], from Thailand.

(ii) Inner capsid protein (VP6). A maximum likelihood phylogenetic tree of VP6-encoding genes, constructed using the Tamura 3 model with gamma distribution and invariant sites, is shown in Fig. 2. Whereas the I5 genotype almost exclusively contained pig RVA strains, the I1 genotype contained mainly human strains. Belgian pig I5 strains were highly related to each other (95.2 to 97.8%). An exception was strain 12R022, which was less related to other Belgian contemporary strains (93.3 to 94.1%) and clustered with Thai strains (94.1 to 94.4%). RV277 and Gottfried clustered within the I1 genotype, together with modern human, archival human, pig, and pig-like human strains. RV277 clustered more closely to archival human strains from the United States (94.0 to 94.5%), a pig strain from China (93.1%), and pig-like human strains from Hungary (93.3 to 93.6%) than to contemporary human strains (89.6 to 91.2%). The genetic distance between RV277 and Gottfried was relatively high (89.3%). Genotype-specific amino acid (aa) mutations between I1 and I5 strains were situated mainly at the interaction site between the VP6 trimer and VP7 (Fig. 3). No genotype I14 strains were encountered in Belgium during the present study.

(iii) Core scaffold protein (VP2) and viral enzymes (VP1 and VP3). The maximum likelihood trees for VP1 and VP2 were constructed using the general time-reversible model with gamma distribution and invariant sites, whereas the Tamura 3 model with gamma distribution and invariant sites was applied for VP3. Phylogenetic analysis of the genes encoding structural proteins VP1, VP2, and VP3 showed that all Belgian porcine RVA strains could be assigned to genotype 1 (Fig. 2). However, while multiple lineages existed within genotype 1 of the corresponding genes, the porcine and human RVAs could mainly be assigned to typical porcine and human subclusters. However, the VP1 and VP2 genes of some archival human Wa-like strains clustered closely with those of pig strains.

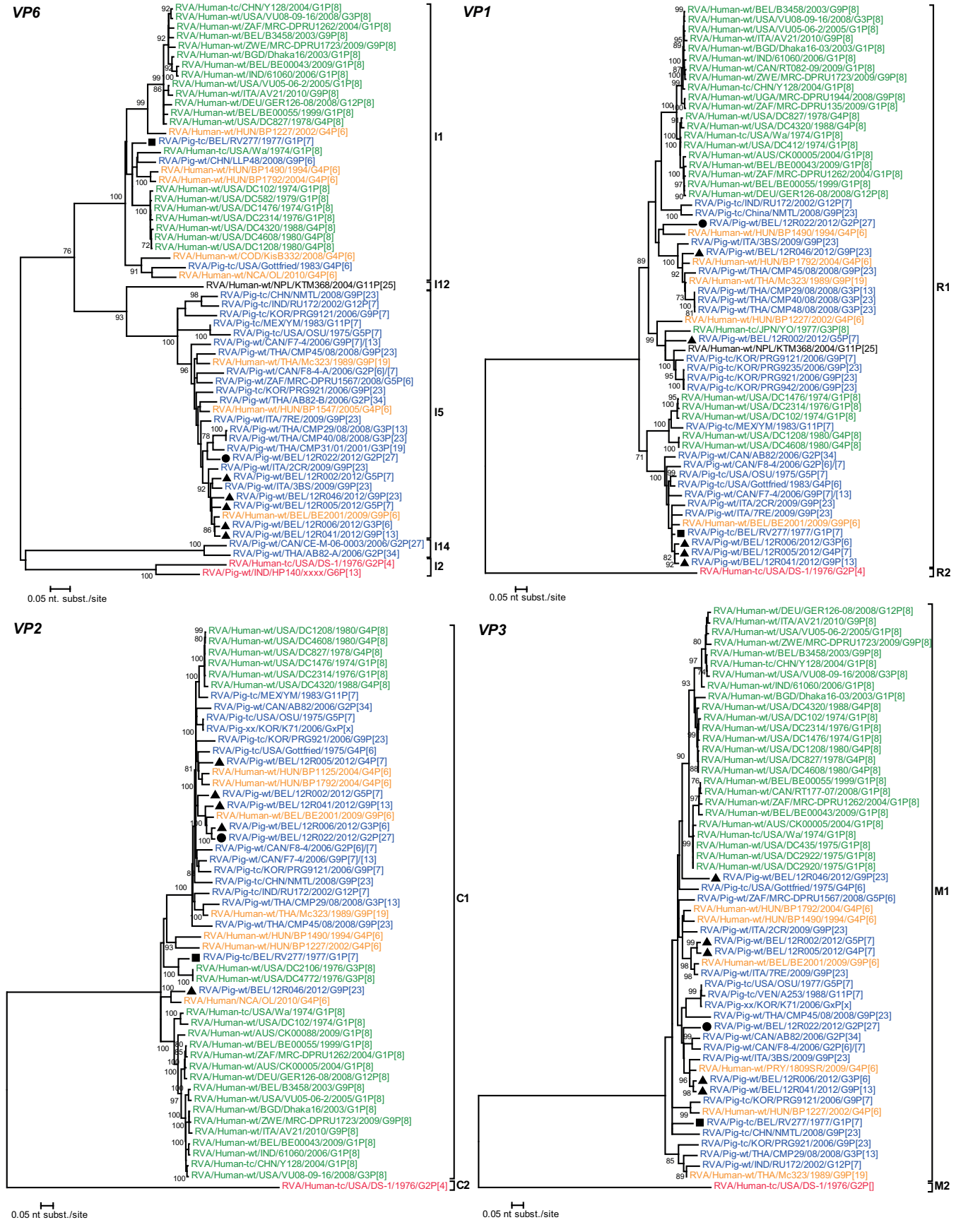

FIG 2 Maximum likelihood phylogenetic trees based on coding sequences of VP6, VP1, VP2, and VP3 genes. Bootstrap values ($n = 500$ replicates) of <70% are not shown. Pig strains are shown in blue, whereas human Wa-like strains are shown in green. Human DS-1-like strains are shown in red. Strains shown in orange are results of suspected interspecies transmission events between pigs and humans. Belgian pig strains are marked with triangles (contemporary and diarrheic), circles (contemporary and nondiarrheic), or squares (diarrheic and historic).

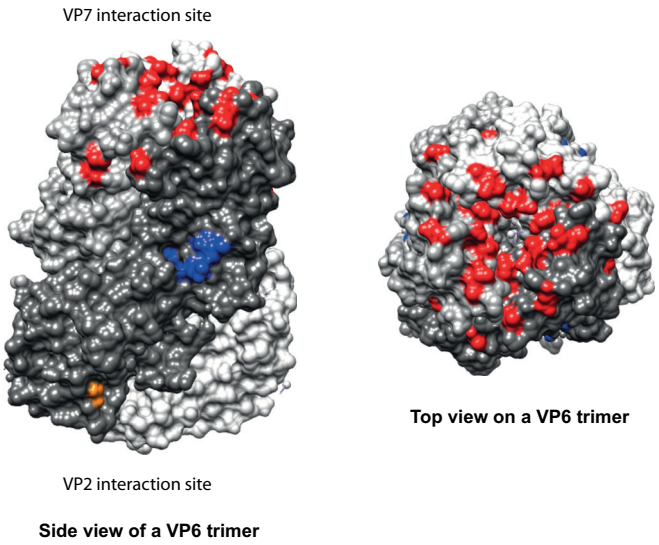

FIG 3 Three-dimensional representation of a VP6 trimer (PDB entry 1QHD) demonstrating genotype-specific amino acid changes between strains of the I1 and I5 genotypes. Mutations at the interaction site between VP6 and VP7 are shown in red. Mutations at the side of VP6 are shown in blue, and mutations at the interaction site with VP2 are shown in orange.

Within the C1 genotype of VP2, Belgian strains 12R002, 12R006, 12R022, and 12R041 formed a subcluster (92.1 to 97.7%) with pig-like human strain BE2001 (92.8 to 95.3%). In contrast, these Belgian strains were genetically more distinct from another Belgian strain, 12R005 (90.8 to 92.1%). Remarkably, pig strain YM, from Mexico, was most closely related to several archival human Wa-like strains from the United States (94.7 to 95.4%). Nonetheless, historic strain RV277 was also closely related to archival human Wa-like strains RVA/Human-wt/USA/DC2106/1976/G3P[8] and RVA/Human-wt/USA/DC4772/1976/G3P[8] (93.0%). In contrast, contemporary human Wa-like strains clustered distinctly from porcine strains. Furthermore, Belgian strain 12R046 clustered most closely to strain RVA/Human-wt/NCA/OL/2010/G4P[6], from Nicaragua (93.3%).

Genes encoding the viral RNA-dependent RNA polymerase (VP1) were classified into two large subclusters within genotype R1. Overall, a high genetic diversity was present among pig strains within this genotype, which was lower between contemporary human strains and between most archival human strains. Belgian strains 12R002, 12R022, and 12R046 belonged to the first clade but were genetically distinct, since they all belonged to different subclusters (85.6 to 86.4%). Within the second clade, Belgian pig strains 12R005, 12R006, 12R041, and RV277 were closely related to each other (96.3 to 97.0%) and formed a subcluster with pig strains from Italy (93.7 to 95.4%) and the pig-like human strain BE2001, from Belgium (95.0 to 96.1%). Remarkably, pig strain YM clustered closely to some archival human Wa-like strains (92.4 to 93.9%).

For the viral guanylyl- and methyltransferase (VP3), all Belgian pig strains belonged to genotype M1, which was composed of different intragenotypic lineages. Pig strains were distinct from contemporary and ancient Wa-like human rotaviruses. Strains 12R002 and 12R005 were genetically closely related to each other (97.5%) and formed a cluster with pig-like human strains and pig strains from Italy (90.7 to 94.7%). Strains 12R006 and 12R041 formed a cluster with Italian and Canadian pig strains and a pig-like human strain from Paraguay (90.7 to 95.2%). Historic strain RV277 was distantly related to the Korean strain RVA/Pig-tc/KOR/PRG9121/2006/G9P[7] (88.8%) and the ancient American pig strain Gottfried (88.8%).

Phylogenetic analysis of genes encoding nonstructural proteins. (i) Interferon antagonist (NSP1). A general time-reversible model was used for construction of the NSP1 maximum likelihood phylogenetic tree. Analysis of the genes encoding the interferon antagonist NSP1 showed the clustering of all Belgian pig strains within the A8 genotype (Fig. 4). Different subclusters were demonstrated for this genotype, but even the genetic distances between strains from the same clusters were highly variable. Belgian strains 12R005, 12R006, and 12R046 formed a subcluster with pig-like human strains from Belgium and Hungary and with the ancient strain Gottfried (89.2 to 97.9%). Furthermore, Belgian strains 12R041, 12R002, and 12R022 clustered with the pig Italian strain RVA/Pig-wt/ITA/2CR/2009/G9P[23] (90.9 to 92.8%). Ancient strain RV277 clustered most closely to pig strains from Italy and Thailand (92.2 to 92.6%). All human and archival Wa-like strains clustered in genotype A1, distinct from some pig strains and the human reference strain Wa.

(ii) Viroplasm-associated proteins (NSP2 and NSP5). The Tamura 3 model with gamma distribution and invariant sites was used for construction of maximum likelihood phylogenetic trees for NSP2 and NSP5. Analysis of the NSP2 genes demonstrated 2 clades within the N1 genotype of NSP2 (Fig. 5). In one subcluster, Belgian strains 12R005 and 12R041 clustered with pig strains from Canada and South Korea and with the rhesus monkey strain RVA/Rhesus-tc/USA/TUCH/2002/G3P[24] (93.6 to 94.5%).

Within the other clade, ancient strain RV277 clustered with pig strains from Thailand, Mexico, and South Korea and with the contemporary Belgian human strain RVA/Human-wt/BEL/B3458/2003/G9P[8] (94.3 to 96.1%). Strain 12R006 clustered most closely to pig-like Brazilian human strain RVA/Human-wt/BRA/HST327/1999/G4P[6] (93.2%). However, this strain was also distantly related to contemporary and archival human Wa-like strains (90.6 to 92.2%) and the pig strain NMTL (91.3%). Another subcluster was formed by Belgian pig strains 12R002, 12R022, and 12R046, which were highly related to the Belgian pig-like human strain BE2001 (96.7 to 97.4%). Remarkably, modern and archival human Wa-like strains could be assigned to different subclusters but were rather distinct from pig strains.

Phylogenetic analysis of the genes encoding NSP5 demonstrated an overall low genetic diversity within genotype H1 (Fig. 5). Pig strain 12R002 clustered with pig strain RVA/Pig-wt/IND/HP140/xxxx/G6P[13] (97.9%) and pig-like human strain RVA/Human-wt/IND/RMC321/xxxx/G9P[19] (97.7%) but was more distantly related to archival human Wa-like strains and one modern Wa-like strain, RVA/Human-wt/AUS/CK00005/2004/G1P[8] (95.4 to 95.7%). Belgian strains 12R005, 12R006, 12R022, 12R041, 12R046, and RV277 were more closely related to each other (96.4 to 98.9%), while being slightly more distantly related to strain 12R002 (95.6 to 96.4%). Belgian pig strains were relatively closely related to another cluster of contemporary and historic human Wa-like strains (94.5 to 98.2%).

(iii) Translation enhancer (NSP3). NSP3 maximum likelihood trees were constructed using the Tamura 3 model with gamma distribution. Genes encoding the NSP3 proteins of Belgian pig RVA strains clustered in two genotypes: T1 and T7

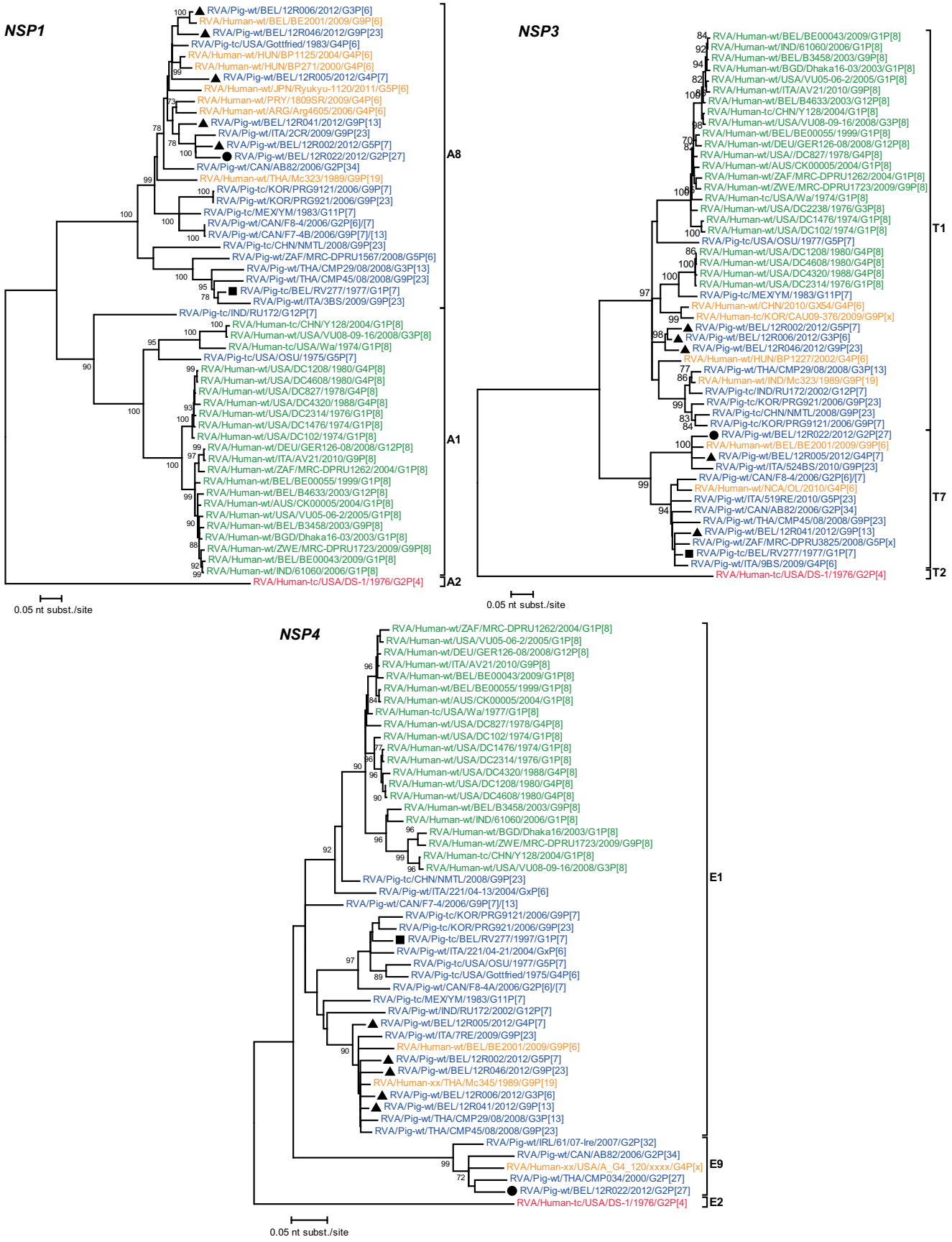

FIG 4 Maximum likelihood phylogenetic trees based on coding sequences of NSP1, NSP3, and NSP4 genes. Bootstrap values ($n = 500$ replicates) of <70% are not shown. Pig strains are shown in blue, whereas human Wa-like strains are shown in green. Human DS-1-like strains are shown in red. Strains shown in orange are results of suspected interspecies transmission events between pigs and humans. Belgian pig strains are marked with triangles (contemporary and diarrheic), circles (contemporary and nondiarrheic), or squares (diarrheic and historic).

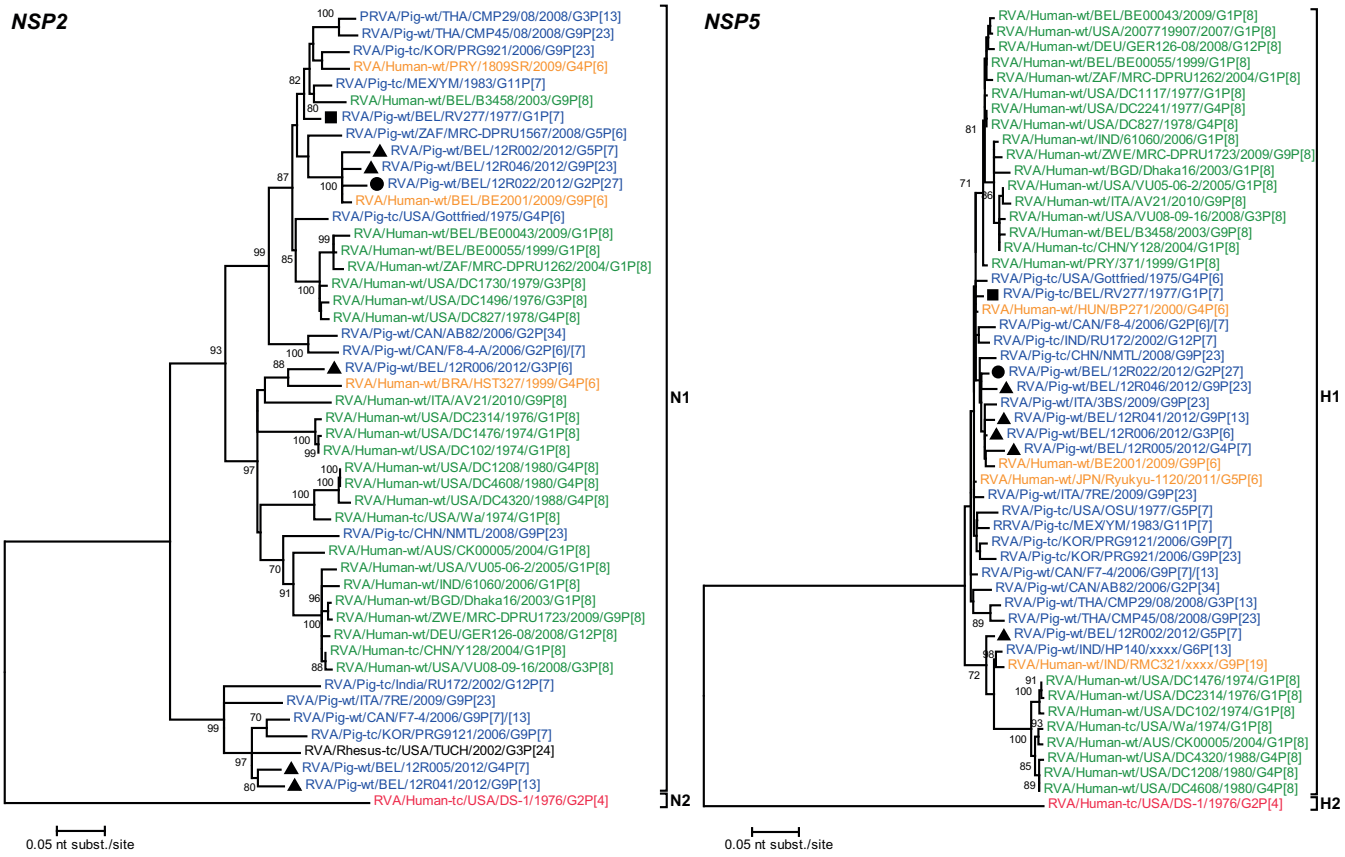

FIG 5 Maximum likelihood phylogenetic trees based on coding sequences of NSP2 and NSP5 genes. Bootstrap values (n = 500 replicates) of <70% are not shown. Pig strains are shown in blue, whereas human Wa-like strains are shown in green. Human DS-1-like strains are shown in red. Strains shown in orange are results of suspected interspecies transmission events between pigs and humans. Belgian pig strains are marked with triangles (contemporary and diarrheic), circles (contemporary and nondiarrheic), or squares (diarrheic and historic).

(Fig. 4). Within genotype T1, a subcluster was formed by Belgian strains 12R002, 12R006, and 12R046 (94.4 to 96.0%). Belgian pig strains within the T1 genotype were more distantly related to modern human Wa-like strains (87.2 to 91.6%) and to a smaller cluster of human archival Wa-like strains from the United States (88.8 to 90.5%). Historic pig strains OSU and YM were relatively distantly related to strains of these modern (86.2 to 88.6%) and archival (88.2 to 92.9%) clusters, respectively. Within the T7 genotype, two phylogenetic clusters were formed. One consisted of the Belgian strains 12R005, 12R022, and BE2001 and the Italian strain RVA/Pig-wt/ITA/524BS/2010/G9P[23] (93.1 to 95.2%), whereas the other cluster within T7 consisted of pig strains 12R041 and RV277, which were moderately related to pig strains from Canada, Italy, Thailand, and South Africa (91.2 to 95.6%).

(iv) Enterotoxin (NSP4). For construction of NSP4 maximum likelihood trees, the Tamura 3 model with gamma distribution was used. Most of the Belgian pig RVA strains clustered within the E1 genotype of NSP4 (Fig. 4). As such, strains 12R002, 12R005, 12R006, 12R041, and 12R046 formed a cluster with strains from Thailand and Italy and with pig-like human strains BE2001 and Mc345 (93.7 to 97.9%). Strain RV277 was only distantly related to the other Belgian pig E1 strains (88.7 to 89.8%), and it clustered with ancient strains OSU and Gottfried and with pig strains from Canada, Italy, and South Korea (93.1 to 94.6%). A major human Wa-like subcluster containing modern and ancient strains was demonstrated for the E1 genotype.

Strain 12R022 belonged to a cluster of pig RVAs from Ireland, Thailand, and Canada within the E9 genotype (90.8 to 92.9%). Only one human strain (RVA/Human-wt/USA/A_G4_120/xxxx/G4P[x]) possessing this genotype has been detected so far, and it was relatively closely related to the pig E9 strains (91.4 to 92.7%).

Analysis of antigenic regions of VP7 of porcine and human strains. An amino acid analysis of the neutralization epitopes present on VP7 of Belgian pig RVA strains was performed. Amino acid residues were compared with corresponding residues of other pig strains and a selection of Belgian human RVA strains (Fig. 6). For VP7, 3 neutralizing domains have been described in the literature, namely, 7-1a, 7-1b, and 7-2 (23, 24). Only 4 of 29 aa (positions 98, 104, 201, and 264) in these antigenic regions were conserved among all pig and human RVA strains included in the present study. Ancient strain RV277 and human strain AU-19 differed at 3 aa positions. An identical number of mutations was noticed between RV277 and the other pig G1 strains. RV277 was antigenically slightly more divergent from contemporary Belgian human G1 strains from lineages I and II, with 4 and 7 aa differences, respectively. Most variable positions were located in epitope 7-1a, but the single most variable residue was located in epitope 7-2 (aa 217). More antigenic differences were present on

	7-1a													7-1b						7-2								
	87	91	94	96	97	98	99	100	104	123	125	129	130	291	201	211	212	213	238	242	143	145	146	147	148	190	217	221	264
G1																													
RVA/Pig-tc/BEL/RV277/1977/G1P[7]	T	N	N	G	E	W	K	D	Q	S	V	V	D	K	Q	N	V	D	N	T	K	D	Q	D	L	S	I	N	G
RVA/Human-tc/JPN/AU19/1997/G1P[6]	I	N	N	G	E	W	K	D	Q	S	V	V	D	K	Q	N	V	D	N	T	K	D	Q	N	L	S	I	D	G
RVA/Pig-wt/GBR/SW20/21/1976/G1P[7]	T	N	N	G	E	W	K	D	Q	S	V	V	D	K	Q	N	V	D	D	T	K	D	Q	N	L	S	V	N	G
RVA/Pig-tc/ARG/C95/xxxx/G1P[x]	V	N	N	G	E	W	K	D	Q	N	V	V	D	K	Q	N	V	D	N	T	K	D	Q	N	L	S	I	N	G
RVA/Human-wt/BEL/BE1286/2009/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R	Q	N	V	D	N	T	K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BEL/BE1128/ 2009/G1P[8]	T	T	S	G	E	W	K	D	Q	N	V	V	D	R	Q	N	T	D	N	T	K	D	Q	N	L	S	T	N	G
RVA/Human-wt/BEL/BE1175/2009/ G1P[8]	T	T	N	G	E	W	K	N	Q	S	V	V	D	K	Q	N	V	D	N	T	K	D	Q	N	L	S	M	N	G
RVA/Human-wt/BEL/BE1280 /2009/G1P[8]	T	T	N	G	E	W	K	D	Q	S	V	V	D	K	Q	N	V	D	N	T	K	D	Q	N	L	S	M	N	G
G2																													
RVA/Pig-wt/BEL/12R022/2012/G2P[27]	T	N	S	T	E	W	T	N	Q	N	A	I	D	.	Q	E	V	N	S	S	R	D	T	T	S	D	T	S	G
RVA/Pig-wt/ESP/34461-4/2003/G2P[23]	T	N	S	T	E	W	T	N	Q	N	A	I	N	K	Q	E	V	N	S	N	K	D	T	T	S	D	T	S	G
RVA/Pig-wt/THA/CMP034/2000/G2P[27]	T	N	S	T	E	W	E	S	Q	N	A	I	N	K	Q	E	V	N	S	S	R	D	T	T	S	D	T	S	G
RVA/Pig-wt/CAN/AB82/2006/G2P[34]	T	N	S	T	E	W	E	N	Q	N	A	I	N	K	Q	E	V	N	S	S	R	D	T	T	S	N	T	S	G
RVA/Pig-wt/CAN/F8-4/2006/G2P[6][7]	T	N	A	T	E	W	E	S	Q	D	N	I	N	K	Q	D	V	S	T	N	R	D	N	N	T	D	V	S	G
RVA/Human-wt/BEL/BE1141/2009/G2P[4]	T	N	S	N	E	W	E	N	Q	D	T	M	N	K	Q	D	V	D	N	S	R	D	N	T	S	D	I	S	G
RVA/Human-wt/BEL/BE1058/2008/G2P[4]	T	N	S	N	E	W	E	N	Q	D	T	M	N	K	Q	D	V	D	N	N	R	D	N	T	S	D	I	S	G
RVA/Human-wt/BEL/BE1251/2009/G2P[4]	T	N	S	N	E	W	E	N	Q	D	A	M	N	K	Q	D	V	D	N	N	R	D	N	T	S	D	I	S	G
RVA/Human-wt/BEL/BE1248/2009/G2P[4]	A	N	S	N	E	W	E	N	Q	D	A	I	N	K	Q	D	V	D	N	S	R	D	N	T	S	D	I	S	G
G3																													
RVA/Pig-wt/BEL/12R006/2012/G3P[6]	T	T	N	N	S	W	K	D	Q	D	A	I	D	K	Q	D	T	N	S	N	K	D	A	A	L	S	E	A	G
RVA/Pig-wt/THA/CMP29/08/2008/G3P[13]	T	T	N	N	S	W	K	D	Q	D	A	V	D	K	Q	D	T	N	S	N	K	D	A	A	L	S	E	A	G
RVA/Pig-wt/THA/CMP096/2001/G3P[19]	T	T	N	N	S	W	K	D	Q	D	A	V	D	K	Q	D	T	N	S	N	K	D	A	A	L	S	E	A	G
RVA/Human-wt/BEL/BE1214/2009/G3P[8]	T	T	N	N	S	W	K	D	Q	D	A	V	D	K	Q	D	T	N	N	N	K	D	A	T	L	S	E	A	G
RVA/Human-wt/BEL/BE1259/2009/G3P[8]	T	T	N	N	S	W	K	D	Q	D	A	V	D	K	Q	D	T	N	N	N	K	D	A	T	L	S	E	A	G
RVA/Human-wt/BEL/BE1322/2009/ G3P[8]	T	T	N	N	S	W	K	D	Q	D	A	V	D	K	Q	D	T	N	N	N	K	D	V	T	L	S	E	D	G
G4																													
RVA/Pig-wt/BEL/12R005/2012/G4P[7]	A	T	G	N	E	W	K	D	Q	N	L	V	D	R	Q	N	T	G	D	A	R	I	A	G	E	S	M	N	G
RVA/Pig-tc/USA/Gottfried/1983/G4P[6]	S	T	N	N	E	W	K	D	Q	N	L	I	D	K	Q	N	T	A	D	T	R	A	S	G	E	S	T	S	G
RVA/Pig-wt/JPN/FGP65/2009/G4P[x]	T	T	S	N	E	W	K	D	Q	N	L	V	D	K	Q	N	T	N	D	T	R	A	S	G	E	S	T	S	G
RVA/Pig-wt/JPN/GUB88/2006/G4P[x]	T	T	S	N	E	W	K	D	Q	N	L	V	D	K	Q	N	T	N	D	T	R	A	S	G	E	S	T	S	G
RVA/Pig-xx/USA/O-1/xxxx/G4P[x]	A	T	G	N	E	W	K	D	Q	N	L	V	D	R	Q	N	T	G	D	T	R	I	S	G	E	S	M	N	G
RVA/Human-wt/BEL/BE1129/2009/G4P[8]	T	T	S	T	E	W	K	D	Q	N	L	I	E	K	Q	N	A	A	D	T	K	T	S	G	E	S	T	S	G
RVA/Human-wt/BEL/BE1113/2009/G4P[8]	T	T	S	T	E	W	K	D	Q	N	L	I	E	K	Q	N	T	A	D	T	K	T	S	G	E	S	T	S	G
G5																													
RVA/Pig-wt/BEL/12R002/2012/G5P[7]	N	T	A	T	K	W	T	E	Q	D	A	V	E	R	Q	D	I	N	D	S	K	D	G	N	L	S	T	A	G
RVA/Pig-tc/USA/OSU/1976/G5P[7]	N	T	A	N	K	W	T	E	Q	D	A	V	E	R	Q	D	K	N	D	N	K	D	G	N	L	S	L	A	G
RVA/Pig-wt/ITA/334/04-1/2004/G5P[27]	N	T	A	T	K	W	T	E	Q	D	A	V	E	R	Q	D	K	N	D	N	K	D	G	N	L	S	T	A	G
RVA/Pig-wt/KOR/I-1/2006/G5P[7]	N	T	A	T	K	W	T	E	Q	D	A	V	E	R	Q	D	I	N	D	S	K	D	G	N	L	S	T	A	G
RVA/Pig-wt/THA/CMP178/200x/G5P[13]	N	T	A	N	K	W	T	E	Q	D	A	I	E	R	Q	D	T	S	D	N	K	D	V	N	L	S	T	A	G
G9																													
RVA/Pig-wt/BEL/12R041/2012/G9P[13]	T	T	G	T	E	W	K	N	Q	N	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	N	G
RVA/Pig-wt/BEL/12R046/2012/G9P[23]	T	T	G	T	E	W	K	N	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	N	G
RVA/Pig-wt/BEL/12R003/2012/G9P[23]	T	T	G	A	K	W	K	D	Q	D	A	I	N	.	Q	N	A	A	D	N	K	D	S	T	L	S	E	R	G
RVA/Pig-wt/BEL/13R068/2013/G9P[7]	T	T	G	A	E	W	K	D	Q	D	A	I	N	.	Q	N	A	A	D	N	K	D	S	T	L	S	E	G	G
RVA/Human-wt/BEL/BE2001/2009/G9P[6]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	R	G
RVA/Pig-wt/THA/CMP45/08/2008/G9P[23]	T	T	G	T	E	W	K	N	Q	N	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	N	G
RVA/Pig-tc/KOR/PRG921/2006/G9P[23]	T	T	G	T	E	W	K	N	Q	D	A	I	D	K	Q	N	K	A	D	N	K	D	S	T	L	S	E	N	G
RVA/Pig-wt/JPN/Hokkaido 14/2001/G9P[23]	T	T	G	A	E	W	K	D	Q	N	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	N	G
RVA/Pig-wt/ITA/2CR/2009/G9P[23]	A	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	N	G
RVA/Human-wt/BEL/BE1242/2009/G9P[8]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
RVA/Human-wt/BEL/BE1119/2009/G9P[8]	T	T	G	T	E	W	K	N	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
RVA/Human-wt/BEL/BE1032/2008/G9P[8]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G

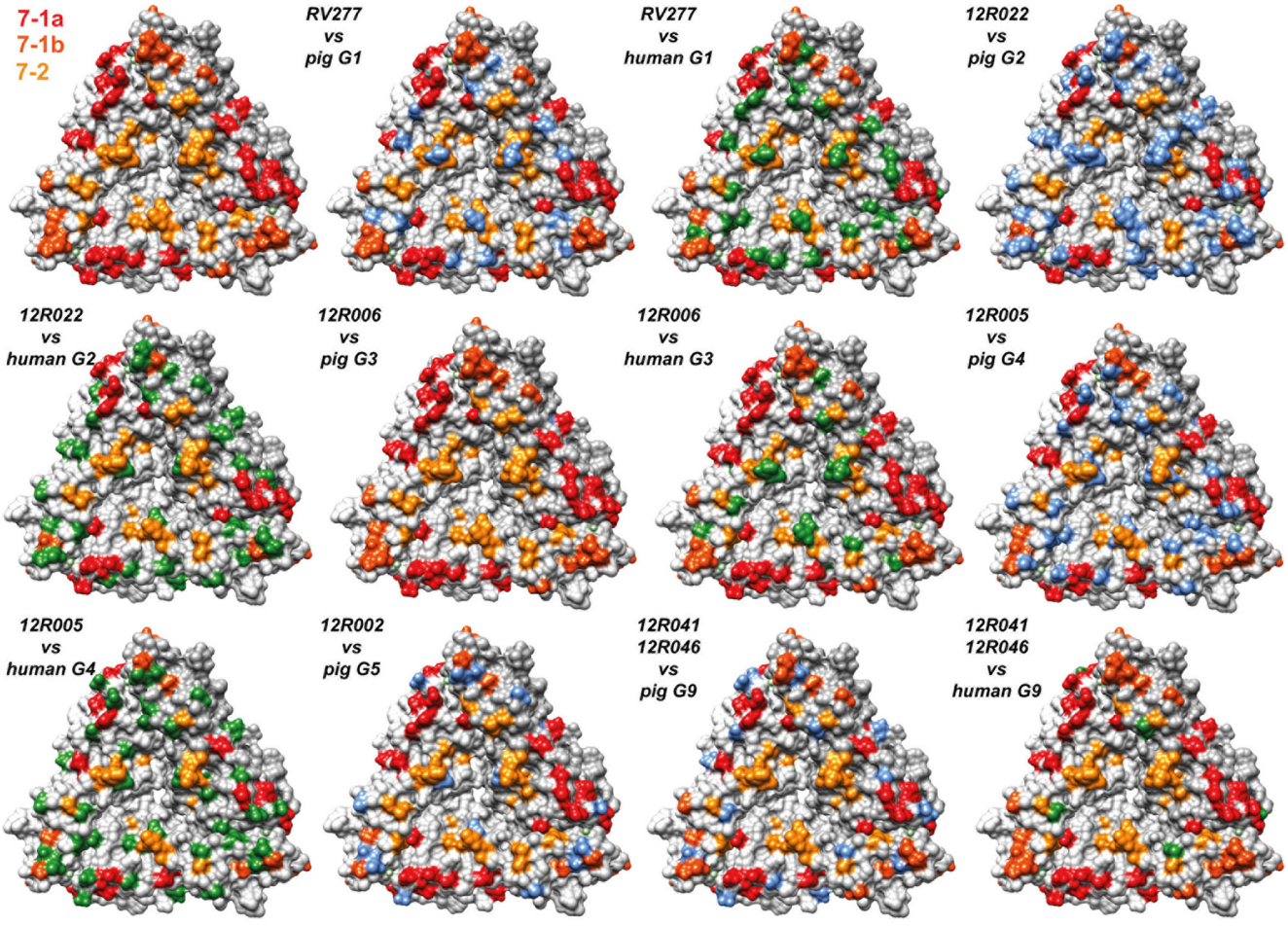

FIG 6 Amino acid residues of the main antigenic regions of genotypes G1 to G5 and G9 of the glycoprotein VP7. (Top) Amino acid mutations between Belgian pig strains from the present study (bold) and pig strains from other countries are shown in blue, whereas mutations between these Belgian pig strains and human strains are shown in green. (Bottom) The main antigenic regions are demonstrated by shades of red and orange in a three-dimensional structural model of the VP7 trimer (PDB entry 3FMG) (model in the upper left corner). The other models are comparisons between Belgian strains and pig/human strains belonging to the same genotype. Mutations are indicated with the same colors as in the upper panel.

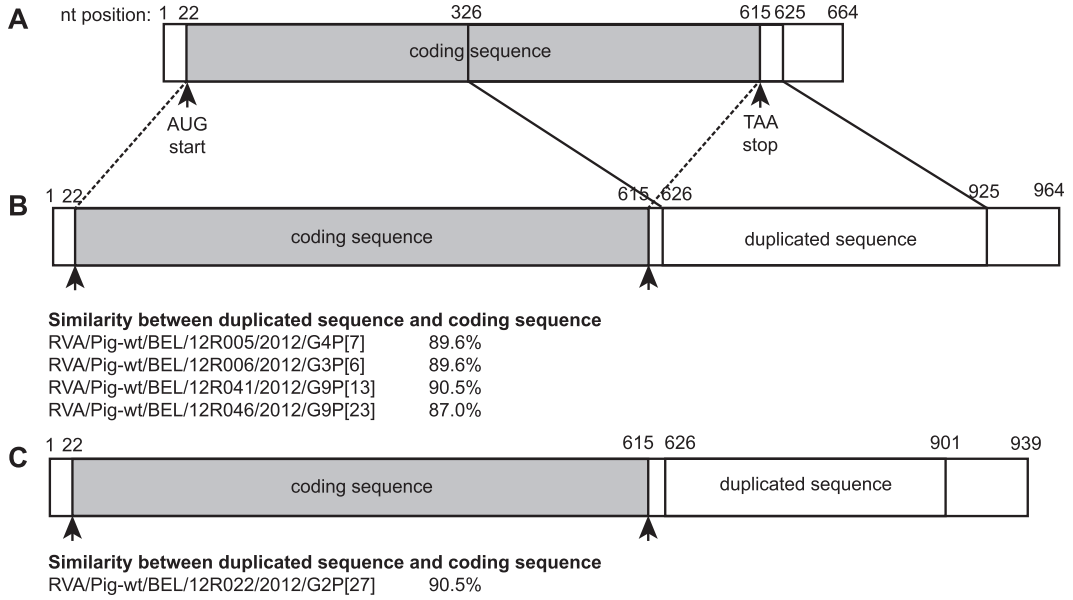

FIG 7 Representation of gene duplications present in the NSP5 genes of pig RVA strains. (A) Normal NSP5 gene segment. (B and C) NSP5 genes of Belgian pig strains containing gene duplications with lengths of 300 and 275 bp, respectively. Start and stop codons are marked with arrows. The coding sequence for NSP5 is shown in gray, whereas noncoding sequences are shown in white.

the heavily glycosylated VP7 proteins belonging to the G2 genotype. Up to 11 aa mutations were present between Belgian pig G2 strain 12R022 and contemporary human G2 strains. Multiple aa differences between pig and human G2 strains were located at possible glycosylation sites. Strain F8-4, which clustered phylogenetically between pig G2 strains and human G2 strains, was different in 14 aa residues from strain 12R022. Within the G3 genotype, antigenic epitopes were relatively conserved among pig and human strains. Only one aa difference (aa 129) was observed between Belgian and Thai pig G3 strains. Three to five aa differences were seen between strain 12R006 and Belgian human G3 strains. In contrast to pig strains, human G3 strains possessed a potentially extra N-linked glycosylation site at aa 238. Two to 10 aa differences were noticed between strain 12R005 and other pig G4 strains. Strain 12R005 was also clearly antigenically different from contemporary human G4 strains, with up to 10 aa differences. Furthermore, pig G5 strains seemed to possess relatively conserved antigenic epitopes, with only two to five aa differences between 12R005 and other pig G5 strains.

The antigenic epitopes of Belgian pig G9 strains 12R041 and 12R046 were nearly identical, with only one aa difference (aa 123). These strains were antigenically more distinct from Belgian strains 12R003 and 13R068, belonging to another phylogenetic lineage within the G9 genotype, with up to seven aa differences. The loss of a possible N-linked glycosylation site in epitope 7-2 (aa 221) was apparent in pig strains 12R003 and 13R068 and in Belgian pig-like human strain BE2001. This N-linked glycosylation was also lacking in the contemporary Belgian G9 strains. Further analysis of the antigenic epitopes showed a high level of antigenic relatedness of pig G9 strains 12R041 and 12R046 and contemporary human G9 strains, with only one other mutation present in epitope 7-1a. Two possible N-linked glycosylation sites, at aa 211 and 242, were conserved among all pig and human G9 strains.

Head-to-tail gene duplications in gene segment 11. Belgian pig strains 12R005, 12R006, 12R022, 12R041, and 12R046 possessed a gene duplication at the 3′ end of gene segment 11 (Fig. 7). The starting point of each duplication was nucleotide (nt) 326 of the parental strain. The duplications of strains 12R005, 12R006, 12R041, and 12R046 were 300 nt long, whereas the duplication of strain 12R022 was 275 nt long. All duplications covered the 3′ end of the NSP5 open reading frame. Overall, similarities between the coding sequences and duplications were relatively low, with 87.0 to 90.5% identity.

DISCUSSION

Group A rotaviruses are considered an important cause of diarrhea in suckling and weaned piglets and are the leading cause of diarrhea in children under 5 years of age. An evolutionary relationship between pig RVAs and human Wa-like strains has been suggested to exist, but there are still many gaps in our understanding of the genetic constellations of pig RVAs (6). Furthermore, it is not completely clear if pig RVAs form a considerable risk for spread in the human population after interspecies transmission and if they can readily reassort with human strains. In the present study, the genetic constellations of six contemporary Belgian pig RVAs and one historic RVA strain were assessed, and their evolutionary relationships with human and other pig RVAs were investigated.

Sequence analyses of the VP7 and VP4 genes of the Belgian strains included in this study showed high levels of genetic diversity in these genes (1). Furthermore, many mutations were present in the antigenic regions of pig strains belonging to different genotypes of VP7. Whether these mutations also result in a reduced cross-protection between different genotypes should be investigated further by sero-neutralization assays using pig intestinal epithelial cells instead of MA104 cells, since the latter do not allow the propagation of all pig strains. VP7 genotypes shared between human and pig RVAs have clearly evolved in different evolution-

ary directions. Indeed, this is demonstrated by the phylogenetic distinction between pig and human strains in different subclusters within the same genotype. These conclusions could also be drawn upon analysis of the amino acid residues of the main antigenic regions, 7-1a, 7-1b, and 7-2, present on this glycosylated outer capsid protein. Especially for G2, human and pig strains clustered phylogenetically separately, and numerous amino acid differences were present in the main antigenic regions of their VP7 proteins. Similar findings were described by others, and it was suggested that pig G2 strains can be categorized as borderline G2 strains, which was further confirmed here (25, 26). In contrast, contemporary pig and human G9 strains seem to be much more related than pig and human strains from other VP7 genotypes. This is also clear from the limited number of mutations present between antigenic regions of pig and human VP7 proteins. Pig G9 strains have been proposed to be ancestors of currently circulating G9 strains in humans. Most likely, these strains adapted to the human population after an interspecies transmission event that occurred relatively recently, in the early 1990s (27). This relatively short time has not enabled the human G9 strains to evolve far from their pig counterparts.

Phylogenetic analysis of the VP4 genes demonstrated the clustering of the major human VP4 genotypes P[8] and P[4] with genotypes P[6] and P[19], which are both shared between humans and pigs (28). As shown in the phylogenetic trees in the present study, almost all pig-like human RVA strains possessed a P[6] genotype for VP4. It can be suggested that pig strains bearing the P[6] genotype cross the species barrier more easily than do strains bearing typical porcine VP4 genotypes, such as P[7]. The VP8* domain of the VP4 protein contains a lectin domain which is responsible for binding to carbohydrates present on mucus and/or the enterocyte's surface. A plausible explanation for a seemingly possible weaker interspecies barrier for P[6] strains is the recognition of a common or similar carbohydrate moiety on pig and human enterocytes by P[6] strains. This might be the H type 1 antigen, which has been demonstrated to be recognized by P[6] strains *in vitro* (29). In contrast, P[7] strains, described as sialidase sensitive, and thus dependent on sialic acids for their attachment to the cell surface *in vitro*, most likely do not fit the carbohydrate moieties present in the human gut, and interspecies transmission events from these typical pig strains to humans are practically never seen (30).

Despite the high genetic diversity seen in the VP7 and VP4 genes, the genetic backbone of Belgian pig RVA strains was relatively conserved. A genetic backbone with an I5-R1-C1-M1-A8-N1-T7-E1-H1 constellation was mainly detected in Belgian pig RVAs, which is similar to genetic constellations detected in pig RVAs from other studies of different geographical locations (Table 2). This conserved backbone might be essential for efficient viral replication of pig RVAs in pig enterocytes. An even more conserved genetic backbone is seen in human RVAs. Two major genogroups of human RVAs circulate worldwide: Wa-like RVAs and DS-1-like RVAs. Whereas Wa-like RVAs predominantly possess internal genotype 1 genes (I1-R1-C1-M1-A1-N1-T1-E1-H1), the DS-1-like strains mainly possess internal genotype 2 genes (I2-R2-C2-M2-A2-N2-T2-E2-H2) (15). Gene reassortments between Wa-like and DS-1-like RVAs have been detected, but not frequently, and it has been suggested that certain gene constellations function best when kept together (15, 31, 32). The classification of the pig rotavirus genes VP1, VP2, VP3, NSP2, NSP4, and NSP5 into genotypes R1, C1, M1, N1, E1, and H1, respectively, which also contain human Wa-like RVAs, is suggestive of an evolutionary relationship between pig and human RVAs. Nonetheless, the present results also indicate that pig and human Wa-like RVAs have entered distinct evolutionary routes. As demonstrated in the phylogenetic trees for VP3, NSP2, NSP4, and NSP5, genes of modern and archival human Wa-like strains are moderately to poorly related to those of pig strains, despite the fact that they belong to the same genotype. Exceptions in the NSP2 tree were two modern human Wa-like strains, B3458 and AV21, which clustered distinctly from other human strains but slightly closer to pig strains. However, for the VP1, VP2, and NSP3 genes, some pig strains were moderately related to archival human Wa-like strains, which was also observed in other studies (33, 34). Furthermore, the genetic diversity between pig genes is usually higher than that between human Wa-like RVAs. It might be that pig RVAs have been circulating longer in the pig population and that they have accumulated more mutations in their viral genes than the cases for human RVA strains. However, whether pig RVAs were progenitors of human RVA strains or *vice versa* cannot be concluded from the present study, but the fact that both species share an ancestor is further confirmed by the phylogenetic relationships of most internal gene segments (6). Whether pig and human Wa-like RVA strains are able to create more viable reassortants than Wa-like and DS-1-like human strains cannot be definitely concluded but can be assumed given the shorter genetic distance between pig and human Wa-like genes. Another explanation for the higher genetic diversity in the pig genes might be the existence of a stronger evolutionary drift in pig RVAs than in human RVAs. This is not unlogical, as pigs repeatedly come into contact with different rotavirus strains during the intense production stages (35, 36).

This stronger evolutionary drift may also be represented by the existence of the I5, A8, and T7 genotypes for VP6, NSP1, and NSP3, respectively. It is possible that proteins encoded by these genotypes allow for a better match with the cellular machinery in the porcine enterocyte during viral replication, but other discrete changes in other viral proteins may be involved in host range restriction as well. Nonetheless, these assumptions should be proven by *in vitro* infection experiments using (reassortant) pig and human RVA strains in relevant cell models of pig and human enterocytes.

NSP1 has already been described to likely be involved in host range restriction (37, 38). Amino acid analysis of NSP1 A1 and A8 strains revealed that the N-terminal domain of the protein, which contains a zinc finger motif, was most conserved among both genotypes. This part of NSP1 functions as an E3 ligase that is necessary for ubiquitination of interferon-regulatory proteins, such as IRF3, IRF5, IRF7, and β-TrCP. These regulatory factors are recognized by the C-terminal half of NSP1. Recognition of these regulatory factors by NSP1 results in ubiquitination of these proteins, followed by proteosomal degradation and suppression of the interferon response (31, 39). However, the C-terminal half of NSP1 was highly variable between the 2 genotypes, and it might be that A8 strains recognize and suppress different proteins, or species variants of these proteins, that are involved in the interferon pathway from those of A1 strains *in vivo*.

While I5 is the dominantly detected genotype for VP6, the I1 genotype is only seldom seen in pigs. In the present study, ancient strain RV277 from 1977 also possessed an I1 genotype for VP6. Furthermore, the I1 genotype was also recently detected in pig-

like human strains from Hungary and the Democratic Republic of Congo (40, 41). Several amino acid changes were observed at the apical site of the VP6 trimer (Fig. 3), which is known to interact with the VP7 outer capsid protein (31). It might be hypothesized that the I5 genotype encodes a viral inner capsid protein that allows a better functional fit with the vastly diverse VP7 and VP4 proteins borne by pig RVA strains.

More and more T7 genotypes of NSP3 are being encountered in pigs nowadays, whereas in the past, only T1 genotypes were detected (17, 18, 20). Genotype-specific aa changes between T1 and T7 strains, but also species-specific amino acid changes not related to the genotype, were observed. Most of these changes were located in the C-terminal region of NSP3, which is known to interact with eukaryotic initiation factor 4G (eIF4G) (31, 42, 43). These species- and genotype-specific changes may lead to a better recognition of human or pig eIF4G, thus likely suppressing host cell protein synthesis in favor of viral protein translation.

Another interesting finding was the detection of an E9 genotype for nondiarrheic strain 12R022. Until now, almost all E9 genotypes have been encountered in pigs. An exception is the detection of an E9 genotype in A_G4_120, a human G4 strain whose VP4 gene was untypeable. Most likely, this strain was the result of a pig-to-human interspecies transmission event. It might be interesting to further investigate and compare the biological functions of NSP4 proteins encoded by E9 and E1 genotypes in pig intestinal epithelial cells.

One of the most conserved genes in pig RVAs was NSP5. This gene is involved in the formation of the viroplasm, but its full repertoire of functions remains to be elucidated. Five Belgian RVA strains possessed a partial head-to-tail gene duplication, with the duplicated regions starting at identical locations at the 3′ end of gene segment 11. An almost identical duplication was also noticed in the Belgian pig-like human strain BE2001, isolated from a child (20). Most duplications were 300 nt long, but that of strain 12R022 was 275 nt long. The presence of these rearrangements behind the stop codon results in an unaltered NSP5 protein. Most likely, these rearranged strains were already circulating for a long time in the Belgian pig population, since relatively low nucleotide identities between the coding sequences and their duplicated counterparts were observed. This suggests the accumulation of many mutations in these duplications over time. The exact function(s) of these duplications remains elusive, but gene segments possessing duplications are thought to be incorporated preferentially into progeny viruses. This principle has therefore been applied to improve rotavirus reverse genetics systems (44).

Regarding the future development of preventive measures against RVA infections in pigs, the high levels of diversity seen for VP7 and VP4 might be a tremendous challenge at first sight. To protect offspring during the first weeks of life, neutralizing lactogenic antibodies directed against different serotypes of VP7 and VP4 are necessary. As such, the development of a multivalent vaccine covering the different G/P types circulating in the pig population might be necessary. In this case, each individual strain will likely elicit a homotypic neutralizing antibody response. Combining multiple strains will then result in broad heterotypic lactogenic protection. In contrast, if a vaccine needs to be applied to young pigs to protect them actively against weaning diarrhea, it might be sufficient to include a monovalent attenuated vaccine, as it has been shown that subsequent rotavirus infections will induce protection against heterotypic strains in humans (45). Internal RVA proteins, such as VP6, VP2, NSP2, and NSP4, also induce nonneutralizing antibodies, but they might have a protective effect *in vivo* (46, 47). VP6, for instance, is one of the most immunogenic proteins in RVA, and anti-VP6 antibodies may have intracellular neutralizing capacities (48). As VP6, VP2, NSP2, and NSP4 are relatively conserved among pigs, the inclusion of a single strain in a weaning piglet vaccine might be sufficient to protect against strains bearing different VP7-VP4 combinations but having similar internal backbones. Further research will be executed to explore these hypotheses.

In conclusion, the evolutionary relationship between pig and human Wa-like RVAs can be confirmed. However, it seems that viruses from both species have entered different evolutionary routes, likely resulting in better adaptation to their host species. This evolutionary diversification might hamper efficient spreading of pig strains in the human population after interspecies transmission, and *vice versa*. Nonetheless, the first species barrier is the interaction of VP4 and carbohydrates on mucus and the surfaces of enterocytes. Because until now almost all pig-like human strains contained a P[6] genotype, it can be concluded that the species barrier is less strict for this genotype. Future surveillance of pig and human RVAs is warranted to facilitate the detection of interspecies transmission events and the occurrence of gene reassortments and subsequent adaptations of pig RVAs to the human population.

ACKNOWLEDGMENTS

M.Z. and I.D.M.R. were supported by doctoral fellowships from the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT Vlaanderen). L.M.B.D. was supported by a doctoral fellowship from the Research Foundation-Flanders (FWO Vlaanderen).

REFERENCES

1. **Theuns S, Desmarets LM, Heylen E, Zeller M, Dedeurwaerder A, Roukaerts ID, Van Ranst M, Matthijnssens J, Nauwynck HJ.** 2014. Porcine group A rotaviruses with heterogeneous VP7 and VP4 genotype combinations can be found together with enteric bacteria on Belgian swine farms. Vet Microbiol **172:**23–34. http://dx.doi.org/10.1016/j.vetmic.2014.04.002.
2. **Matthijnssens J, Otto PH, Ciarlet M, Desselberger U, Van Ranst M, Johne R.** 2012. VP6-sequence-based cutoff values as a criterion for rotavirus species demarcation. Arch Virol **157:**1177–1182. http://dx.doi.org/10.1007/s00705-012-1273-3.
3. **Saif LJ, Jiang B.** 1994. Nongroup A rotaviruses of humans and animals. Curr Top Microbiol Immunol **185:**339–371.
4. **Molinari BL, Lorenzetti E, Otonel RA, Alfieri AF, Alfieri AA.** 2014. Species H rotavirus detected in piglets with diarrhea, Brazil, 2012. Emerg Infect Dis **20:**1019–1022. http://dx.doi.org/10.3201/eid2006.130776.
5. **Marthaler D, Rossow K, Culhane M, Goyal S, Collins J, Matthijnssens J, Nelson M, Ciarlet M.** 2014. Widespread rotavirus H in commercially raised pigs, United States. Emerg Infect Dis **20:**1203–1206. http://dx.doi.org/10.3201/eid2007.140034.
6. **Matthijnssens J, Ciarlet M, Heiman E, Arijs I, Delbeke T, McDonald SM, Palombo EA, Iturriza-Gomara M, Maes P, Patton JT, Rahman M, Van Ranst M.** 2008. Full genome-based classification of rotaviruses reveals a common origin between human Wa-like and porcine rotavirus strains and human DS-1-like and bovine rotavirus strains. J Virol **82:**3204–3219. http://dx.doi.org/10.1128/JVI.02257-07.
7. **Matthijnssens J, Ciarlet M, Rahman M, Attoui H, Banyai K, Estes MK, Gentsch JR, Iturriza-Gomara M, Kirkwood CD, Martella V, Mertens PP, Nakagomi O, Patton JT, Ruggeri FM, Saif LJ, Santos N, Steyer A, Taniguchi K, Desselberger U, Van Ranst M.** 2008. Recommendations for the classification of group A rotaviruses using all 11 genomic RNA segments. Arch Virol **153:**1621–1629. http://dx.doi.org/10.1007/s00705-008-0155-1.
8. **Matthijnssens J, Ciarlet M, McDonald SM, Attoui H, Banyai K, Brister**

JR, Buesa J, Esona MD, Estes MK, Gentsch JR, Iturriza-Gomara M, Johne R, Kirkwood CD, Martella V, Mertens PP, Nakagomi O, Parreno V, Rahman M, Ruggeri FM, Saif LJ, Santos N, Steyer A, Taniguchi K, Patton JT, Desselberger U, Van Ranst M. 2011. Uniformity of rotavirus strain nomenclature proposed by the Rotavirus Classification Working Group (RCWG). Arch Virol **156:**1397–1413. http://dx.doi.org/10.1007/s00705-011-1006-z.

9. **Trojnar E, Sachsenroder J, Twardziok S, Reetz J, Otto PH, Johne R.** 2013. Identification of an avian group A rotavirus containing a novel VP4 gene with a close relationship to those of mammalian rotaviruses. J Gen Virol **94:**136–142. http://dx.doi.org/10.1099/vir.0.047381-0.
10. **Martella V, Banyai K, Matthijnssens J, Buonavoglia C, Ciarlet M.** 2010. Zoonotic aspects of rotaviruses. Vet Microbiol **140:**246–255. http://dx.doi.org/10.1016/j.vetmic.2009.08.028.
11. **Papp H, Laszlo B, Jakab F, Ganesh B, De Grazia S, Matthijnssens J, Ciarlet M, Martella V, Banyai K.** 2013. Review of group A rotavirus strains reported in swine and cattle. Vet Microbiol **165:**190–199. http://dx.doi.org/10.1016/j.vetmic.2013.03.020.
12. **Debouck P, Pensaert M.** 1979. Experimental infection of pigs with Belgian isolates of the porcine rotavirus. Zentralbl Veterinarmed B **26:**517–526.
13. **Matthijnssens J, Rahman M, Ciarlet M, Zeller M, Heylen E, Nakagomi T, Uchida R, Hassan Z, Azim T, Nakagomi O, Van Ranst M.** 2010. Reassortment of human rotavirus gene segments into G11 rotavirus strains. Emerg Infect Dis **16:**625–630. http://dx.doi.org/10.3201/eid1604.091591.
14. **Ghosh S, Kobayashi N, Nagashima S, Chawla-Sarkar M, Krishnan T, Ganesh B, Naik TN.** 2010. Full genomic analysis and possible origin of a porcine G12 rotavirus strain RU172. Virus Genes **40:**382–388. http://dx.doi.org/10.1007/s11262-010-0454-y.
15. **Matthijnssens J, Van Ranst M.** 2012. Genotype constellation and evolution of group A rotaviruses infecting humans. Curr Opin Virol **2:**426–433. http://dx.doi.org/10.1016/j.coviro.2012.04.007.
16. **Kim HH, Matthijnssens J, Kim HJ, Kwon HJ, Park JG, Son KY, Ryu EH, Kim DS, Lee WS, Kang MI, Yang DK, Hyun BH, Park SI, Park SJ, Cho KO.** 2012. Full-length genomic analysis of porcine G9P[23] and G9P[7] rotavirus strains isolated from pigs with diarrhea in South Korea. Infect Genet Evol **12:**1427–1435. http://dx.doi.org/10.1016/j.meegid.2012.04.028.
17. **Martel-Paradis O, Laurin MA, Martella V, Sohal JS, L'Homme Y.** 2013. Full-length genome analysis of G2, G9 and G11 porcine group A rotaviruses. Vet Microbiol **162:**94–102. http://dx.doi.org/10.1016/j.vetmic.2012.08.028.
18. **Monini M, Zaccaria G, Ianiro G, Lavazza A, Vaccari G, Ruggeri FM.** 2014. Full-length genomic analysis of porcine rotavirus strains isolated from pigs with diarrhea in northern Italy. Infect Genet Evol **25:**4–13. http://dx.doi.org/10.1016/j.meegid.2014.03.024.
19. **Okitsu S, Khamrin P, Thongprachum A, Kongkaew A, Maneekarn N, Mizuguchi M, Hayakawa S, Ushijima H.** 2013. Whole-genomic analysis of G3P[23], G9P[23] and G3P[13] rotavirus strains isolated from piglets with diarrhea in Thailand, 2006–2008. Infect Genet Evol **18:**74–86. http://dx.doi.org/10.1016/j.meegid.2013.05.005.
20. **Zeller M, Heylen E, De Coster S, Van Ranst M, Matthijnssens J.** 2012. Full genome characterization of a porcine-like human G9P[6] rotavirus strain isolated from an infant in Belgium. Infect Genet Evol **12:**1492–1500. http://dx.doi.org/10.1016/j.meegid.2012.03.002.
21. **Lambden PR, Cooke SJ, Caul EO, Clarke IN.** 1992. Cloning of noncultivatable human rotavirus by single primer amplification. J Virol **66:**1817–1822.
22. **Maes P, Matthijnssens J, Rahman M, Van Ranst M.** 2009. RotaC: a Web-based tool for the complete genome classification of group A rotaviruses. BMC Microbiol **9:**238. http://dx.doi.org/10.1186/1471-2180-9-238.
23. **Aoki ST, Settembre EC, Trask SD, Greenberg HB, Harrison SC, Dormitzer PR.** 2009. Structure of rotavirus outer-layer protein VP7 bound with a neutralizing Fab. Science **324:**1444–1447. http://dx.doi.org/10.1126/science.1170481.
24. **McDonald SM, Matthijnssens J, McAllen JK, Hine E, Overton L, Wang S, Lemey P, Zeller M, Van Ranst M, Spiro DJ, Patton JT.** 2009. Evolutionary dynamics of human rotaviruses: balancing reassortment with preferred genome constellations. PLoS Pathog **5:**e1000634. http://dx.doi.org/10.1371/journal.ppat.1000634.
25. **Martella V, Ciarlet M, Baselga R, Arista S, Elia G, Lorusso E, Banyai K, Terio V, Madio A, Ruggeri FM, Falcone E, Camero M, Decaro N, Buonavoglia C.** 2005. Sequence analysis of the VP7 and VP4 genes identifies a novel VP7 gene allele of porcine rotaviruses, sharing a common evolutionary origin with human G2 rotaviruses. Virology **337:**111–123. http://dx.doi.org/10.1016/j.virol.2005.03.031.
26. **Collins PJ, Martella V, Buonavoglia C, O'Shea H.** 2010. Identification of a G2-like porcine rotavirus bearing a novel VP4 type, P[32]. Vet Res **41:**73. http://dx.doi.org/10.1051/vetres/2010045.
27. **Matthijnssens J, Heylen E, Zeller M, Rahman M, Lemey P, Van Ranst M.** 2010. Phylodynamic analyses of rotavirus genotypes G9 and G12 underscore their potential for swift global spread. Mol Biol Evol **27:**2431–2436. http://dx.doi.org/10.1093/molbev/msq137.
28. **Liu Y, Huang P, Tan M, Liu Y, Biesiada J, Meller J, Castello AA, Jiang B, Jiang X.** 2012. Rotavirus VP8*: phylogeny, host range, and interaction with histo-blood group antigens. J Virol **86:**9899–9910. http://dx.doi.org/10.1128/JVI.00979-12.
29. **Huang P, Xia M, Tan M, Zhong W, Wei C, Wang L, Morrow A, Jiang X.** 2012. Spike protein VP8* of human rotavirus recognizes histo-blood group antigens in a type-specific manner. J Virol **86:**4833–4843. http://dx.doi.org/10.1128/JVI.05507-11.
30. **Ciarlet M, Ludert JE, Iturriza-Gomara M, Liprandi F, Gray JJ, Desselberger U, Estes MK.** 2002. Initial interaction of rotavirus strains with N-acetylneuraminic (sialic) acid residues on the cell surface correlates with VP4 genotype, not species of origin. J Virol **76:**4087–4095. http://dx.doi.org/10.1128/JVI.76.8.4087-4095.2002.
31. **Heiman EM, McDonald SM, Barro M, Taraporewala ZF, Bar-Magen T, Patton JT.** 2008. Group A human rotavirus genomics: evidence that gene constellations are influenced by viral protein interactions. J Virol **82:**11106–11116. http://dx.doi.org/10.1128/JVI.01402-08.
32. **McDonald SM, McKell AO, Rippinger CM, McAllen JK, Akopov A, Kirkness EF, Payne DC, Edwards KM, Chappell JD, Patton JT.** 2012. Diversity and relationships of cocirculating modern human rotaviruses revealed using large-scale comparative genomics. J Virol **86:**9148–9162. http://dx.doi.org/10.1128/JVI.01105-12.
33. **McDonald SM, Davis K, McAllen JK, Spiro DJ, Patton JT.** 2011. Intra-genotypic diversity of archival G4P[8] human rotaviruses from Washington, DC. Infect Genet Evol **11:**1586–1594. http://dx.doi.org/10.1016/j.meegid.2011.05.023.
34. **Zhang S, McDonald PW, Thompson TA, Dennis AF, Akopov A, Kirkness EF, Patton JT, McDonald SM.** 2014. Analysis of human rotaviruses from a single location over an 18-year time span suggests that protein coadaption influences gene constellations. J Virol **88:**9842–9863. http://dx.doi.org/10.1128/JVI.01562-14.
35. **Miyazaki A, Kuga K, Suzuki T, Kohmoto M, Katsuda K, Tsunemitsu H.** 2013. Annual changes in predominant genotypes of rotavirus A detected in the feces of pigs in various developmental stages raised on a conventional farm. Vet Microbiol **163:**162–166. http://dx.doi.org/10.1016/j.vetmic.2012.11.044.
36. **Miyazaki A, Kuga K, Suzuki T, Tsunemitsu H.** 2012. Analysis of the excretion dynamics and genotypic characteristics of rotavirus A during the lives of pigs raised on farms for meat production. J Clin Microbiol **50:**2009–2017. http://dx.doi.org/10.1128/JCM.06815-11.
37. **Broome RL, Vo PT, Ward RL, Clark HF, Greenberg HB.** 1993. Murine rotavirus genes encoding outer capsid proteins VP4 and VP7 are not major determinants of host range restriction and virulence. J Virol **67:**2448–2455.
38. **Feng N, Yasukawa LL, Sen A, Greenberg HB.** 2013. Permissive replication of homologous murine rotavirus in the mouse intestine is primarily regulated by VP4 and NSP1. J Virol **87:**8307–8316. http://dx.doi.org/10.1128/JVI.00619-13.
39. **Arnold MM, Sen A, Greenberg HB, Patton JT.** 2013. The battle between rotavirus and its host for control of the interferon signaling pathway. PLoS Pathog **9:**e1003064. http://dx.doi.org/10.1371/journal.ppat.1003064.
40. **Papp H, Borzak R, Farkas S, Kisfali P, Lengyel G, Molnar P, Melegh B, Matthijnssens J, Jakab F, Martella V, Banyai K.** 2013. Zoonotic transmission of reassortant porcine G4P[6] rotaviruses in Hungarian pediatric patients identified sporadically over a 15 year period. Infect Genet Evol **19:**71–80. http://dx.doi.org/10.1016/j.meegid.2013.06.013.
41. **Heylen E, Batoko Likele B, Zeller M, Stevens S, De Coster S, Conceicao-Neto N, Van Geet C, Jacobs J, Ngbonda D, Van Ranst M, Matthijnssens J.** 2014. Rotavirus surveillance in Kisangani, the Democratic Republic of the Congo, reveals a high number of unusual genotypes and gene segments of animal origin in non-vaccinated symptomatic children. PLoS One **9:**e100953. http://dx.doi.org/10.1371/journal.pone.0100953.
42. **Piron M, Delaunay T, Grosclaude J, Poncet D.** 1999. Identification of the

RNA-binding, dimerization, and eIF4GI-binding domains of rotavirus nonstructural protein NSP3. J Virol **73:**5411–5421.

43. **Piron M, Vende P, Cohen J, Poncet D.** 1998. Rotavirus RNA-binding protein NSP3 interacts with eIF4GI and evicts the poly(A) binding protein from eIF4F. EMBO J **17:**5811–5821. http://dx.doi.org/10.1093/emboj/17.19.5811.
44. **Troupin C, Dehee A, Schnuriger A, Vende P, Poncet D, Garbarg-Chenon A.** 2010. Rearranged genomic RNA segments offer a new approach to the reverse genetics of rotaviruses. J Virol **84:**6711–6719. http://dx.doi.org/10.1128/JVI.00547-10.
45. **Velazquez FR, Matson DO, Calva JJ, Guerrero L, Morrow AL, Carter-Campbell S, Glass RI, Estes MK, Pickering LK, Ruiz-Palacios GM.** 1996. Rotavirus infections in infants as protection against subsequent infections. N Engl J Med **335:**1022–1028. http://dx.doi.org/10.1056/NEJM199610033351404.
46. **Svensson L, Sheshberadaran H, Vene S, Norrby E, Grandien M, Wadell G.** 1987. Serum antibody responses to individual viral polypeptides in human rotavirus infections. J Gen Virol **68:**643–651. http://dx.doi.org/10.1099/0022-1317-68-3-643.
47. **Desselberger U, Huppertz HI.** 2011. Immune responses to rotavirus infection and vaccination and associated correlates of protection. J Infect Dis **203:**188–195. http://dx.doi.org/10.1093/infdis/jiq031.
48. **Aiyegbo MS, Sapparapu G, Spiller BW, Eli IM, Williams DR, Kim R, Lee DE, Liu T, Li S, Woods VL, Jr, Nannemann DP, Meiler J, Stewart PL, Crowe JE, Jr.** 2013. Human rotavirus VP6-specific antibodies mediate intracellular neutralization by binding to a quaternary structure in the transcriptional pore. PLoS One **8:**e61101. http://dx.doi.org/10.1371/journal.pone.0061101.
49. **Shi H, Chen J, Li H, Sun D, Wang C, Feng L.** 2012. Molecular characterization of a rare G9P[23] porcine rotavirus isolate from China. Arch Virol **157:**1897–1903. http://dx.doi.org/10.1007/s00705-012-1363-2.